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TIME-RESOLVED RESONANCE RAMAN SPECTROSCOPY OF CYTOCHROME *c* REDUCED BY PULSE RADIOLYSIS

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The first investigation of the dynamics of a redox transition of an electron-transfer enzyme by time-resolved resonance Raman spectroscopy in combination with pulse-radiolytical reduction is described by an application to cytochrome *c*. A long-lived transient state is observed upon reduction of the alkaline form of cytochrome *c* as a distinct frequency shift of one resonance Raman band. From the frequency in the stable oxidized state, 1567 cm^{-1}, this particular resonance Raman band shifts within less than 1 μs to 1533 cm^{-1} in the transient reduced state, which has a lifetime longer than 20 ms but shorter than a few seconds. Finally, in the stable reduced state, this band is located at 1547 cm^{-1}. According to a previous normal coordinate analysis, this resonance Raman band can be assigned predominantly to a stretching mode of the outermost C-C bonds in the four pyrrole rings of porphyrin. This vibrational mode is influenced by the protein most directly through the covalent thioether linkages of two cysteines to porphyrin. We interpret the long lifetime of the transient state as due to the slow return of Met-80 as sixth ligand to the heme iron upon reduction of the alkaline form of cytochrome *c*.

Introduction

In elucidating the redox mechanism of electron-transfer enzymes, it is particularly important to establish the existence and dynamics of any protein conformational changes accompanying changes of oxidation state. The existence of slow (milliseconds to seconds) conformational changes with oxidation state alteration has implications for the function of electron-transfer enzymes in biological energy-conversion systems. As the oxidation state of an active center of an electron-transfer enzyme is changed, the protein remains at first instance in a conformation corresponding to the previous oxidation state and starts to relax towards its new equilibrium form. Excited conformational energy released during the relaxation of non-equilibrium conformations of electron-transfer enzymes might be utilized for endergonic reactions such as ATP synthesis. Such a mechanism might be underlying the biochemical descriptions of energy transduction in terms of direct conformational and indirect chemiosmotic coupling [1]. Recent developments of the physical principles of biological energy conversions include thermodynamic arguments for

Although it has been recommended to name cytochrome *c* an electron-transfer protein (Enzyme Nomenclature, Recommendations (1972) of the International Union of Pure and Applied Chemistry and International Union of Biochemistry, ch. 6, Elsevier, Amsterdam, 1973), we find it convenient to name it an electron-transfer enzyme in this discussion of its function. Cytochrome *c* satisfies the definition of an enzyme (ibid) since it catalyzes an electron-transfer reaction. We use the concept protein for the polypeptide chain and thus consider the enzyme as composed of the protein and the heme group, a terminology which is useful in the discussion of protein-heme interactions. The terminology used here also facilitates the application of the mechanisms discussed in this paper to enzyme function in general.

[2] and a quantum-mechanical description of [3] this type of mechanism in which enzymes operate as molecular machines.

The electron-transfer enzyme most thoroughly investigated with respect to the redox mechanism is cytochrome *c* [4]. As an intermediate in the mitochondrial respiratory chain between energy-transducing complexes, cytochrome *c* itself is not required to undergo any energy-transferring conformational transitions, but may serve as a model of some subunits of energy-transducing complexes. Another function of conformational changes with oxidation state is the modulation of binding affinities to cytochrome *c* reductase and oxidase.

Static comparisons of cytochrome *c* in its oxidized and reduced states are based on various spectroscopic measurements on the heme and structure determinations of heme and protein. Applied spectroscopic techniques include optical absorption [5], circular dichroism [6,7], NRM [8], resonance Raman scattering [9,10] and extended X-ray absorption fine structure [11]. EPR is detectable in the oxidized state only [12]. Structural comparisons between oxidation states of cytochrome *c* in crystalline form have been obtained by X-ray crystallography [13–16], and in solution by NMR [8,17–19] and extended X-ray absorption fine structure [11] spectroscopy.

A static non-equilibrium form of an electron-transfer enzyme can be prepared by γ-irradiation of a solution of oxidized enzyme at low temperature. The active center is then reduced by hydrated electrons generated by the γ-irradiation, whereas the protein conformation is locked in its oxidized form at the low temperature. One non-equilibrium form of cytochrome *c*, prepared in this manner, has been analyzed by optical absorption spectroscopy [20,21].

Dynamic studies of redox transitions of cytochrome *c* are performed by observation of the time course of heme spectral properties upon rapid change of the oxidation state. The stopped-flow technique has been combined with detection of optical absorption [22–25], circular dichroism [26,27] and, using rapid freezing, EPR [24]. A better time resolution is provided by the pulse-radiolysis technique, in which hydrated electrons are produced to serve as reductants. The transition of the enzyme has been monitored by optical absorption spectroscopy [28–34].

In the dynamic studies, the detection of protein transitions is based on the influence of the protein on heme spectra. The influence of varying protein conformations due to different amino acid sequences of cytochromes *c* from various species has been studied by optical absorption [35], circular dichroism [6,7], EPR [36], NMR [37] and resonance Raman scattering [38,39]. The way that heme spectra are influenced by changes of protein conformation of cytochrome *c* brought about by variations of pH of the enzyme solution has been studied by optical absorption [40,41], circular dichroism [41], EPR [42], NMR [43,44], resonance Raman scattering [38,45] and extended X-ray absorption fine structure [11]. It has been concluded that resonance Raman spectroscopy provides the proper information for a detailed study of protein-dependent effects on the heme.

Resonance Raman spectroscopy has been applied extensively to biological systems [46–49], in particular to heme proteins. Only a few years ago, however, this technique was applied for the first time to short-lived intermediates [50], an excited triplet state [51], and most recently to biological processes on the nano- and picosecond time scale initiated by laser pulses [52–57]. The present paper describes, by an application to cytochrome *c*, the first investigation of the dynamics of a redox transition of an electron-transfer enzyme by time-resolved resonance Raman spectroscopy in combination with pulse-radiolytical reduction.

Experimental Procedure

The equipment for time-resolved resonance Raman spectroscopy of pulse-radiolytically induced transients has been described in detail earlier [58], and is, therefore, only briefly outlined here in the context of specifying experimental conditions.

The electron accelerator was a FEBETRON 705 B, producing 30-ns pulses of 2 MeV electrons. The electron dose of one pulse was chosen as 70 krad, which corresponds to 0.20 mM of hydrated electrons. The analyzing laser pulse, of 1 μs duration and 10 mJ energy, was generated by a flashlamp-pumped tunable dye laser. The excitation wavelength was tuned to 549.7 nm in resonance with the optical absorption α-band of reduced cytochrome *c* at 550 nm. The time-resolved detection of the resonance Raman spec-

trum was obtained by delaying the laser pulse for a variable time from the electron pulse. After recording the spectrum of transients generated by the electron pulse, the spectrum of the stable reduced form was obtained with a second laser pulse a few seconds later for comparison. A rectangular sample cell of fused silica was anaerobically filled from a syringe and emptied by Ar pressure. Electrons and laser beams were perpendicular and the scattered light was detected at right-angles to both beams and included all polarizations. A spectrograph with a grating of 1200 grooves/mm and removed exit slit permitted 400 cm^{-1} of the spectrum to be observed simultaneously. The spectrum was recorded by an optical multichannel detection unit, consisting of an image intensifier and an SIT TV-camera and was processed by a computer. Each spectrum presented in Figs. 1 and 2 represents an accumulation of five separate measurements, each involving a filling of the sample cell with fresh solution. The intensity plotted at one frequency represents the value of a fourth-degree polynomial fit to the seven closest data points centered at the frequency in question. The reported frequencies are estimated to be accurate to within ±1 TV-channel corresponding to ±2 cm^{-1}.

The cytochrome *c* (Sigma type VI from horse heart) was used without further purification. The proportion of reduced cytochrome *c* present in the solution not irradiated by electrons was estimated to be less than 5% from separate optical absorption measurements. Unless otherwise stated, the following conditions were employed: The concentration of cytochrome *c* was 0.20 mM, which is close to optimal with respect to the resonance Raman spectroscopy. As scavenger of $OH^{\cdot}$ radicals, *tert*-butanol was used at a concentration of 1.3 M (10%, w/w), which ascertains that less than 2 μM of the $OH^{\cdot}$ radicals react with cytochrome *c*. Solutions were prepared with 4-times distilled water and deoxygenated by bubbling with high purity Ar. No additions to adjust pH were made to avoid any influence of extra ions on cytochrome *c*. All experiments were performed at room temperature.

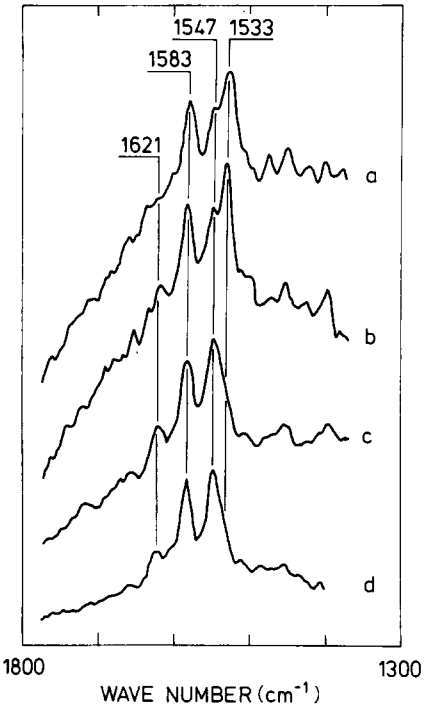

Fig. 1. Resonance Raman spectra in the frequency range 1400–1800 cm^{-1} of cytochrome *c* generated with a 1 μs, 10 mJ, 549.7 nm laser pulse. Spectra a–c were obtained on cytochrome *c* pulse-radiolytically reduced by a 30 ns, 70 krad, 2 MeV electron pulse. The delay time between electron and laser pulse is (a) 11 μs, (b) 20 ms, and (c) a few seconds. Spectrum d is obtained on cytochrome *c* chemically reduced by sodium dithionite. The sample of 0.20 mM cytochrome *c* and 1.3 M *tert*-butanol was prepared in 4-times distilled water and deoxygenated by bubbling with Ar. The pH was 8.2. Each spectrum represents an accumulation of five separate measurements.

Results and Discussion

Fig. 1 shows resonance Raman spectra in the frequency range 1400–1800 cm^{-1} of transient (a, b) and stable (c) forms of pulse-radiolytically reduced and, for comparison, chemically reduced (d) cytochrome *c*. The spectra of the transient forms (a, b) are obtained with a delay time of 11 μs and 20 ms, respectively, between electron and laser pulse, whereas the spectrum of the stable form (c) is obtained with a laser pulse a few seconds after the electron pulse.

One result of our experiments is the verification that oxidized cytochrome *c* in aqueous solution is reduced by pulse-radiolytically produced hydrated electrons. This has previously been concluded from the agreement between optical absorption spectra of

pulse-radiolytically reduced cytochrome *c* and cytochrome *c* chemically reduced by sodium dithionite [28–33]. The resonance Raman spectra contain more information and thus provide a more sensitive test. In the comparison of the resonance Raman spectra, the excitation conditions must be considered. The optical absorption spectrum of cytochrome *c* originates from two allowed porphyrin $\pi \rightarrow \pi^*$ electron transitions of symmetry E_u in the molecular point group D_{4h} [60, 61]. In the reduced state, the weaker of the two bands, the α-band, is located at 550 nm and has an associated vibrational side band, the β-band, at 521 nm, and the stronger band, the γ- or Soret band, is located at 416 nm [5]. Resonance of laser frequency with the α- or β-band enhances Raman scattering from vibrational modes of symmetries B_{1g} and B_{2g} with depolarized scattering and A_{2g} modes with inversely polarized scattering, whereas resonance with the γ-band enhances A_{1g} modes with polarized scattering [10,59]. The frequency of the dye laser used in our experiments was tuned in resonance with the α-band. The resonance Raman spectrum of the stable form of pulse-radiolytically reduced cytochrome *c* in the frequency range 600–1800 cm^{-1} agrees, within experimental accuracy, with respect to band frequencies and relative intensities with the spectrum that we obtain on chemically reduced cytochrome *c* using the same type of excitation. In the comparison with previously published resonance Raman spectra of chemically reduced cytochrome *c* [9,10,59] band frequencies agree. The comparison of band intensities is then not direct because of excitation within the β-band and the polarized detection used previously. Compared to previously reported vidicon detection of continuously [62] as well as pulse-excited [63] resonance Raman spectra of chemically reduced cytochrome *c* the signal-to-noise ratio of the present measurements seems better, which may, however, be due to the different excitation wavelengths used.

The main objective of the present investigation is to study transient states of cytochrome *c* generated upon reduction. The kinetics of the reduction of cytochrome *c* by hydrated electrons have been studied previously by the pulse-radiolysis technique in combination with optical absorption spectroscopy [28–34]. The primary step in the reduction, the bimolecular reaction between oxidized cytochrome *c* and hydrated electrons, is a second-order process with a reaction rate constant which depends on pH and ionic strength [28,30–33]. In a neutral solution of triple-distilled water without a buffer or other ions, the rate constant was determined to be $6 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ [31]. The reduction yield per hydrated electron is about 75% in a neutral solution [30–33]. If we apply these values to our solution of 0.20 mM cytochrome *c* and 0.20 mM hydrated electrons we obtain an estimate of the half-time of this reaction step as 0.1 μs. The primary step is followed by first-order intramolecular processes, interpreted as conformational transitions [28–34]. Regarding the reduction mechanism, it has been concluded that the electrons react mainly with the heme site directly, though some electron transfer may take place via the protein. The fact that the first-order processes appear the same when measured at different wavelengths has led to the conclusion that they do not correspond to intramolecular electron-transfer processes [31,32]. Our delay time between electron and laser pulse of 11 μs is long enough to permit fast (microsecond) first-order processes to die out so that the spectrum obtained, spectrum a of Fig. 1, serves as a starting point for the study of slow (milliseconds to seconds) first-order processes.

The most prominent difference in the Raman spectrum of the transient obtained in the time interval 1 μs to 20 ms compared to that of the stable oxidized and reduced forms of cytochrome *c* is the appearance of a vibrational band at 1533 cm^{-1}. The intensity of this band relative to that at 1547 cm^{-1} (which also appears in the stable reduced form) is a complicated function of different parameters such as pH, ionic strength and concentration of scavenger as seen from Figs. 1 and 2 and discussed below. This band is present less than 1 μs after the electron pulse and lasts at least for 20 ms, but less than a few seconds. In the stable oxidized form of cytochrome *c* the intensities of the resonance Raman bands are lower than in the stable reduced form [9]. They were therefore seen only very weakly under the present experimental conditions. From continuously excited resonance Raman spectra the frequency of the particular resonance Raman band under consideration has previously [38] been determined to vary from 1561 to 1567 cm^{-1} in the stable oxidized form between neutral and alkaline pH.

The experimental conditions employed, which

were chosen for the reasons given in Experimental Procedure, imply that part of the cytochrome *c* is present in its alkaline form. After the establishment of the existence of an alkaline form of cytochrome *c* [22,23,40,41,64], it was shown to correspond to the displacement of Met-80 as the sixth ligand to the heme iron in the oxidized state [65]. What residue replaces Met-80 is not established [24,25,43,66,67], though Lys-79 and Lys-72 are favored candidates. Upon reduction, however, the neutral conformation is more stable even at high pH, and Met-80 returns as sixth ligand [24,68]. A change of oxidation state of the heme is thus accompanied by a considerable conformational change of the protein at alkaline pH. A weak optical absorption band at 695 nm in the oxidized state, originating from a charge-transfer transition, serves as an indicator of the presence of Met-80 as sixth ligand [69–71]. The pK value of the alkaline isomerization of oxidized cytochrome *c* in an aqueous solution varies between 8.9 and 9.3 depending on experimental conditions such as ionic strength [22,24,25,40,41,43,67,72]. However, in the presence of alcohols the pK shifted to lower values [73–75]. From measured titration curves of the 695 nm optical absorption band in 1.3 M *tert*-butanol, the solution used in our pulse-radiolysis experiments, we have determined the pK to be 8.1 at minimal ionic strength, i.e., varying pH by adding NaOH or HCl. The pH value of the solutions used in our pulse-radiolysis experiments was 8.2, corresponding to about 60% of the original oxidized cytochrome *c* present in the alkaline form.

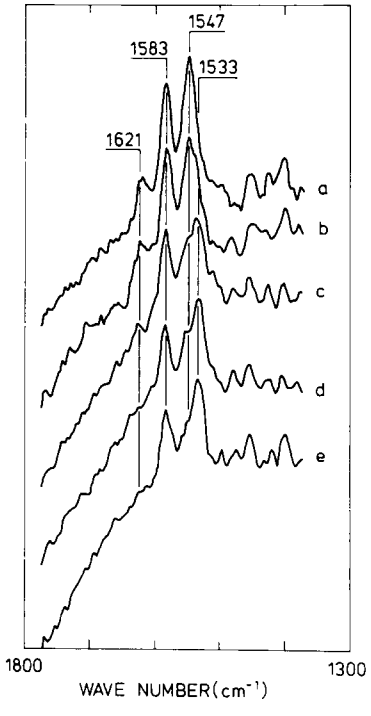

Fig. 2. Resonance Raman spectra in the frequency range 1400–1800 cm^{-1} of pulse-radiolytically reduced cytochrome *c* obtained at a delay time of 11 μs between electron and laser pulse. Characteristics of pulses are as given in Fig. 1. Solutions of 0.20 mM cytochrome *c* were prepared with varying pH, ionic strength and concentration of scavenger and deoxygenated by bubbling with Ar. (a) pH 7.0, 0.05 M sodium phosphate buffer, 0.13 M *tert*-butanol; (b) pH 8.0, 0.05 M sodium phosphate buffer, 0.13 M *tert*-butanol; (c) pH 8.2, no buffer, 0.13 M *tert*-butanol; (d) pH 8.2, no buffer, 1.3 M *tert*-butanol; (e) pH 10.3 by addition of NaOH, 1.3 M *tert*-butanol. Each spectrum represents an accumulation of five separate measurements.

To ascertain that the observed transient state is a non-equilibrium one generated upon reduction of the alkaline form of cytochrome *c*, a series of experiments was performed in which it was investigated how the appearance of the transient state changed as a function of pH, ionic strength and concentration of scavenger. The results are shown in Fig. 2, where all spectra are obtained with a delay time of 11 μs between the electron and laser pulse. No transient state is observed in spectrum a, which is obtained under the same experimental conditions as before, apart from the adjustment of pH to 7.0 with 0.05 M sodium phosphate buffer and the reduction of the concentration of scavenger to 1% (w/w) *tert*-butanol. The increase of pH to 8.0, also by means of 0.05 M sodium phosphate buffer, produces an indication of the existence of a transient state as a shoulder on the 1547 cm^{-1} band, which is shown in spectrum b. Lowering the ionic strength by exclusion of the buffer, whereupon pH takes the value 8.2, enhances the amplitude of the shifted band, as seen in spectrum c. In the next step, the concentration of the scavenger is increased from 1 to 10% (w/w) *tert*-butanol so that spectrum d is identical to spectrum a of Fig. 1. In the last step, the concentration of scavenger is maintained at 10% (w/w) *tert*-butanol, whereas pH is adjusted to 10.3 by addition of NaOH. In this case only a shoulder is present at the frequency 1547 cm^{-1}, whereas the predominant band is

at the shifted frequency 1533 cm^{-1}, as seen in spectrum e. The appearance of the transient state as a function of pH, ionic strength and concentration of scavenger is consistent with the influence of these factors on the appearance of the alkaline form of cytochrome *c* as established by our measurements of the optical absorption band at 695 nm. We thus conclude that the long lifetime of the observed transient state is due to a slow conformational change of the protein permitting return of Met-80 as sixth ligand to the heme iron upon reduction of the alkaline form of cytochrome *c*.

It is consistent with the assignment according to a normal coordinate analysis [76] of the resonance Raman band with frequency 1547 cm^{-1} in the stable reduced state that this vibrational mode is the one most influenced by the protein. The assignment of the band is predominantly a stretching mode of the outermost C-C bonds in the four pyrrole rings of porphyrin. The influence from the protein is transmitted most directly through the covalent thioether linkages of two cysteines to the porphyrin. It has been observed that this resonance Raman band is the most sensitive to replacement of the sixth ligand [38]. The frequency, 1533 cm^{-1}, of this band in the transient reduced state of the alkaline form also coincides with the frequency of the corresponding band in the stable reduced state of the di-carboxymethylated form of cytochrome *c* at alkaline pH [77,78]. The residues carboxymethylated are Met-65 and Met-80 [79], and this modification causes the displacement of Met-80 as sixth ligand to the heme iron [79]. As for the alkaline form, the replacing ligand is not established [78–83], though again Lys-79 is a probable candidate. In the di-carboxymethylated form, the Met-80 remains displaced in the reduced state [79]. The coinciding frequencies of the corresponding resonance Raman bands in the transient reduced state of the native form at alkaline pH and the stable reduced state of the di-carboxymethylated form at alkaline pH might suggest a similarity regarding the molecular nature between these two states. Another way of obtaining information on the molecular nature of the transient state is by comparison with model compounds. The resonance Raman spectrum of ferrous protoporphyrin bis-imidazole shows bands at 1534 and 1583 cm^{-1} [84] similar to those of our transient state.

Another resonance Raman band which, in the transient state 11 μs after the electron pulse, is affected by the presence of alkaline form is located at 1621 cm^{-1}. The series of spectra in Fig. 2 shows a gradual decrease in intensity of this band as the proportion of alkaline form increases. However, as seen in spectrum b of Fig. 1, this band has begun to reappear weakly already 20 ms after the electron pulse. It is therefore possible that the relaxation of the intensity of this band indicates another stage of the protein relaxation process than the one discussed above.

The conformational transition upon reduction of the alkaline form of cytochrome *c* has been detected by optical absorption [22–25], circular dichroism [26,27] and EPR [24] spectroscopy in stopped-flow experiments and by optical absorption spectroscopy in pulse-radiolysis experiments [28–30,32]. The use of resonance Raman spectroscopy to monitor the conformational transition offers more possibilities of detecting the underlying mechanisms.

With the present resolution and signal-to-noise ratio, we have not been able to resolve any resonance Raman frequency shifts within the frequency range 600–1800 cm^{-1} due to the transient state of the neutral form of cytochrome *c*. Cytochrome *c* goes over to the alkaline form in a particular lipid solution, and it has therefore been proposed that the alkaline form and its redox-induced conformational transition might be of relevance to the in vivo situation [85]. However, as pointed out in the Introduction, cytochrome *c* is an intermediate in the mitochondrial respiratory chain, and is not itself required to undergo any large energy-delivering conformational changes with change of oxidation state. Indeed, only small conformational differences between the oxidized and reduced forms have been detected, e.g., by X-ray crystallography [13–16]. The method investigated preliminarily here, viz., time-resolved resonance Raman spectroscopy of pulse-radiolytically reduced electron-transfer enzymes, might become a valuable tool for the study of energy-transducing complexes, where large conformational changes with oxidation state are expected.

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